Differentiation 87 (2014) 161–171

Contents lists available at ScienceDirect

Differentiation

journal homepage: www.elsevier.com/locate/diff

Novel roles of *Pkd2* in male reproductive system development

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ARTICLE INFO

Article history:
Received 5 December 2013
Received in revised form
28 April 2014
Accepted 30 April 2014
Available online 18 June 2014

Keywords:
Polycystin-2
Polycystic kidney disease
Efferent duct
Epididymis
Testis
Mice

ABSTRACT

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common inherited genetic diseases, caused by mutations in *PKD1* and/ or *PKD2*. Infertility and reproductive tract abnormalities in male ADPKD patients are very common and have higher incidence than in the general population. In this work, we reveal novel roles of *Pkd2* for male reproductive system development. Disruption of *Pkd2* caused dilation of mesonephric tubules/efferent ducts, failure of epididymal coiling, and defective testicular development. Deletion of *Pkd2* in the epithelia alone was sufficient to cause reproductive tract defects seen in $Pkd2^{-/-}$ mice, suggesting that epithelial *Pkd2* plays a pivotal role for development and maintenance of the male reproductive tract. In the testis, *Pkd2* also plays a role in interstitial tissue and testicular cord development. In-depth analysis of epithelial-specific knockout mice revealed that *Pkd2* is critical to maintain cellular phenotype and developmental signaling in the male reproductive system. Taken together, our data for the first time reveal novel roles for *Pkd2* in male reproductive system development and provide new insights in male reproductive system abnormality and infertility in ADPKD patients.

1. Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common genetic diseases in humans affecting all ethnic groups worldwide with an incidence up to 1 in 500. Renal pathogenesis of ADPKD is characterized by development of renal cysts, enlargement of kidneys and progressive loss of renal function. Over half of ADPKD patients proceed to end-stage renal disease by the fifth and sixth decades. ADPKD is caused by a spectrum of mutations in *PKD1* on chromosome 16 and/or *PKD2* gene on chromosome 4. *PKD1* mutations account for over 85% of the cases; *PKD2* mutations are responsible for the rest of the cases. Reproductive system abnormalities and infertility in male ADPKD patients are very common and have a higher incidence than in the general population, suggesting that ADPKD genes are required for development or maintenance of the male reproductive system (Belet et al., 2002; Kanagarajah et al., 2011; Manno et al., 2005; Torra et al., 2008; Vora et al., 2008).

The male reproductive system consists of a set of sex organs, including the paired testes and a network of excretory ducts known as the reproductive tract. The male reproductive tract consists of a number of sex accessory organs, including the efferent ducts, epididymis, vas deferens, and seminal vesicle on each side. The efferent ducts, connecting the testis to the epididymis, are formed by mesonephric tubule remodeling, while other structures are mostly derived from the Wolffian duct that degenerates in females (Cornwall, 2009; Hannema and Hughes, 2007; Herpin and Schartl, 2011; Joseph et al., 2009). Testis development is initiated by establishment of a group of specialized epithelial cells during early gonadogenesis, the Sertoli cells, which are derived from the coelomic epithelium at the genital ridge (Brennan and Capel, 2004; Karl and Capel, 1998). The newly emerged Sertoli cells align around aggregates of the primordial germ cells (PGCs), which migrate to the genital ridge at an earlier stage (E8.5 to E9.5 in mice), to form the testicular cords (Brennan and Capel, 2004; Molyneaux and Wylie, 2004). An important function of the Sertoli cells is to secret the anti- Müllerian hormone (AMH) to inhibit development of the female sex organ primordia. The interstitial compartment of the male gonad is formed by mesenchymal cells which migrate from the mesonephros during gonadogenesis (Brennan and Capel, 2004; Combes et al., 2009; Martineau et al., 1997). These mesenchymal cells differentiate into various cell types, including endothelial cells, peritubular myoid cells, and the Leydig cells (Brennan and Capel, 2004; Combes et al., 2009). The testicular cords, after being

Abbreviations: ADPKD, autosomal dominant polycystic kidney disease; HRP, horseradish peroxidase; PC-1, polycystin-1; PC-2, polycystin-2; PGC, primordial germ cell; PKD, polycystic kidney disease; TUNEL, terminal deoxynucleotidyl-transferased UTP nick end labeling

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http://dx.doi.org/10.1016/j.diff.2014.04.001
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formed, continuously grow and convolute, and eventually mature into the seminiferous ducts. Sperm are generated in the testis and mature while migrating through the tortuous and lengthy reproductive tract.

In a previous study, we showed that the *Pkd1* gene plays essential roles for male reproductive tract development (Nie and Arend, 2013). Here, we examined if *Pkd2* is also required for male reproductive system development. The *Pkd2* gene encodes polycystin-2 (PC-2), a membrane protein with six transmembrane domains that are homologous to the last six transmembrane domains of polycystin -1 (PC-1) (Cai et al., 1999; Chapin and Caplan, 2010; Wilson, 2001). Unlike PC-1, the N-terminal and C-terminal of PC-2 are both intracellularly located. PC-2 serves as an ion channel for a variety of cell types and is critical for development of a number of organ systems (Chapin and Caplan, 2010; Gonzalez-Perrett et al., 2001; Koulen et al., 2002; Tsiokas et al., 2007; Wilson, 2001). Multiple lines of evidence demonstrate that PC-1 and PC-2 are co-localized in a variety of cell types. Physical interactions of PC-1 and PC-2 have also been detected at their intracellular domains (Casuscelli et al., 2009; Qian et al., 1997; Xu et al., 2003; Yoder et al., 2002). Further, disruption of *Pkd1* and *Pkd2* in mice results in similar phenotypes in many organ systems. Yet, *Pkd2* also displays unique expression and functions that are not found for *Pkd1*. For example, *Pkd2* is expressed in cells of the embryo node and plays an essential role for body left-right determination, in which *Pkd1* is not required (Bataille et al., 2011;

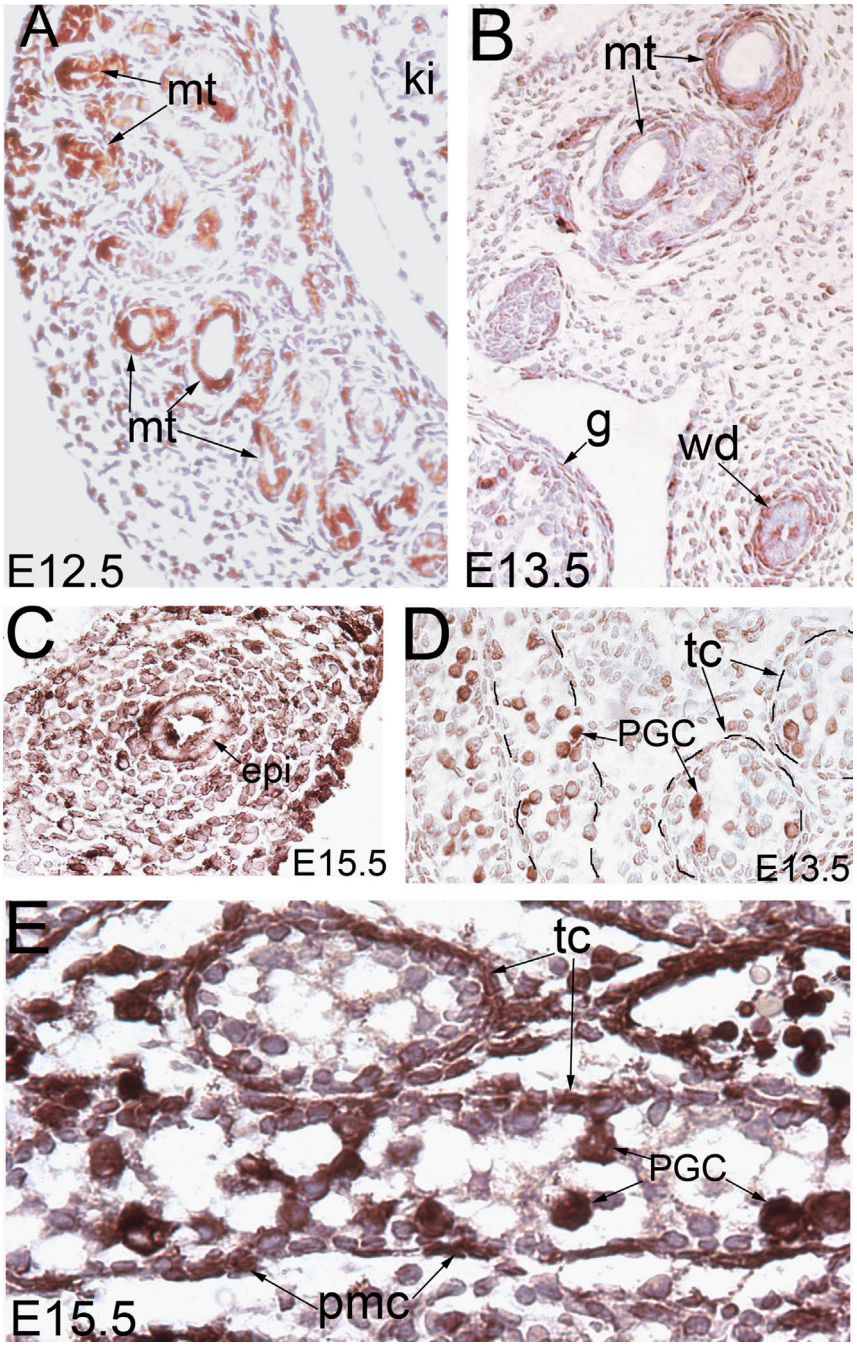

Fig. 1. PC-2 staining in the developing male reproductive system. (A) PC-2 staining of the mesonephros at E12.5. (B) PC-2 staining of the mesonephros and the Wolffian duct at E13.5. (C) PC-2 staining of the epididymis. (D) PC-2 staining of E13.5 testis. (E) PC-2 staining of E15.5 testis. epi: epididymis; g: gonad; ki: kidney; mt: mesonephric tubules; PGC: primordial germ cells; pmc: peritubular myoid cell; tc: testicular cord; wd: Wolffian duct.

Yoshiba et al., 2012). Overall, *Pkd1* and *Pkd2* exert both common and independent functions during organogenesis. In this study, we reveal novel roles for *Pkd2* in male reproductive system development.

2. Material and methods

2.1. Mice

Animal use protocol was approved by Johns Hopkins University Animal Care and Use Committee. *Pkd1*$^{LacZ/+}$(*Pkd1*$^{+/-}$), *Pkd2*$^{LoxP/LoxP}$, *Pkd2*$^{+/-}$ and *Pax2-cre* mice were described elsewhere (Bhunia et al., 2002; Garcia-Gonzalez et al., 2010; Ohyama and Groves, 2004; Wu et al., 2000). The mice were maintained in a mixed background and genotyped by PCR.

2.2. Histology, immunohistochemistry and immunofluorescence

Histology, immunofluorescence and immunohistochemistry were performed using paraffin sections at thickness of 7 μm. Tissue preparation and embedding were performed using standard procedures. Hematoxylin and eosin staining was used for histologic study. Immunohistochemical staining and immunofluorescence were performed using antibodies to PC-2 (Millipore), phospho-Smad2 (Cell signaling Technology), phospho-Smad1/5 (Cell signaling Technology), pan-cytokeratin (Sigma), β-catenin (Sigma), α-tubulin (Neomarkers), and laminin (Sigma). Secondary antibodies were either horseradish peroxidase (HRP) or fluorescence conjugated. Antibody dilution followed manufacturer's recommendations. Diaminobenzidine (DAB) was used for HRP-mediated color reaction.

2.3. Apoptosis and proliferation assays

Paraffin sections were used for apoptosis and proliferation assays. TUNEL staining for apoptosis used the in situ Cell Death Detection Kit (Roche) and was performed following the manufacturer's protocol. The phospho-histone H3 antibody (Millipore) was used for the proliferation assay. The fraction of phospho-histone H3 positive cells over total number of cells was used for proliferation index.

2.4. LacZ staining

Urogenital system primordia were dissected out from mouse embryos, fixed with 4% paraformaldehyde for 1 h at 4 °C, rinsed thrice in 1 × PBS, and stained overnight at room temperature in X-gal reaction solution containing 35 mM potassium ferrocyanide, 35 mM potassium ferricyanide, 2 mM MgCl2, and 1 mg/ml X-gal. Stained tissues were visualized in 80% glycerol or sectioned for microscopic examination.

2.5. RT-PCR and real-time qRT-PCR

Total RNA was isolated from the efferent ducts of three mice for each genotype with Purelink RNA mini kit (Life Technologies) according to the manufacturer's instructions. Reverse transcription was performed using High Capacity RNA-to-DNA kit following the manufacturer's protocol (A&B Applied Biosystems). Primers for *β-catenin* were described elsewhere and confirmed with RT-PCR (Gilbert-Sirieix et al., 2011). The real-time PCR step was performed in a CFX Touch real-time PCR detecting system (Bio-Rad). Fluorescence was acquired at each cycle (40 amplification cycles).

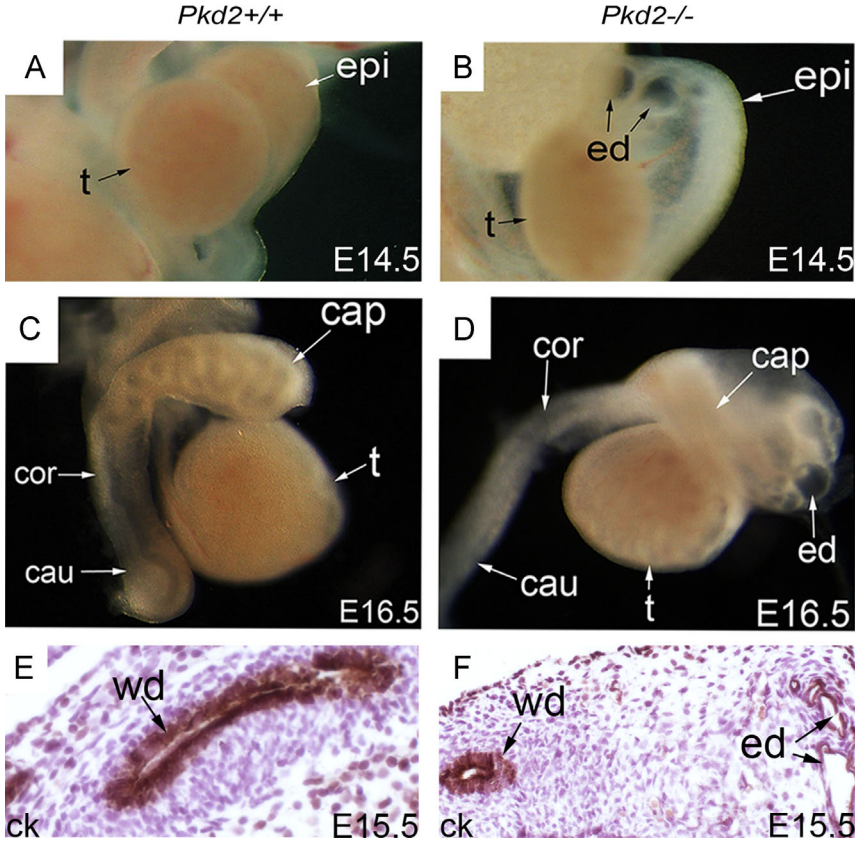

Fig. 2. Male reproductive tract defects in *Pkd2*$^{-/-}$ mice. (A, B) The male reproductive system at E14.5, showing efferent duct dilation in the mutant. (C, D) The male reproductive system at E16.5, showing severe dilation of the efferent ducts and coiling defect of the epididymal duct in the mutant. (E, F) Pan-cytokeratin staining of the Wolfian ducts at E15.5. cap: caput epididymis; cau: cauda epididymis; ck: pan-cytokeratin; cor: corpus epididymis; ed: efferent duct; epi: epididymis; t: testis.

Ct values were normalized to that of GAPDH. Product specificity was confirmed by melting curve analysis.

2.6. Western blot

Frozen tissues of mouse reproductive system were homogenized in RIPA buffer containing protease inhibitors (50 μl RIPA per 1 mg tissue). The homogenates were left on ice for 30 min, then heated at 95 °C for 5 min, and spun down at 11,000 g for 5 min. The supernatants were collected for western blot. About 40 μg proteins were loaded for each genotype. Protein transfer was performed using a semi-dry system. HRP-mediated chemiluminescent signal was detected by a Kodak Digital Image Station 4000R system.

3. Results

3.1. Pkd2 encoded PC-2 expression in the male reproductive system

PC-2 expression was seen in the Wolffian duct and mesonephros in both mesenchyme and epithelium in human tissues (Chauvet et al., 2002). Here, we examined the spatiotemporal expression of *Pkd2* during male reproductive system development by using an antibody for mouse PC-2. We detected PC-2 in the mesonephric tubules at E12.5 (Fig. 1A), consistent with the stage of expression in a human tissue study (Chauvet et al., 2002). One day later, PC-2 expression was reduced in the epithelium but appeared in mesenchyme associated with the Wolffian duct and mesonephric tubules (Fig. 1B and C). In the epithelial cells, it was seen in both luminal side and basal side (Fig. 1B and C).

PC-2 has been detected in the Sertoli cells and germ cells in the testicular cords during postnatal development (Markowitz et al., 1999). However, it is unknown if it is required for embryonic development of the testis. Here, we found that PC-2 was expressed in the PGCs of E13.5 testis (Fig. 1D). By E15.5, it was also seen in the interstitial cells of the testis (Fig. 1E). Altogether, PC-2 showed dynamic expression in multiple tissues in the male reproductive system, suggestive of a developmental role.

3.2. Male reproductive tract defects in $Pkd2^{-/-}$ mice

Next, we examined the male reproductive system in $Pkd2^{-/-}$ mice. Many of the $Pkd2^{-/-}$ mice died beginning at E13.5 to E16.5 with multiple organ system defects. The earliest defect in the male reproductive system of $Pkd2^{-/-}$ mice was mesonephric tubule/efferent duct dilation, which became evident at E13.5 to E15.5 (Fig. 2A and B). The Wolffian ducts of mutant and control mice, labeled by pan-cytokeratin staining, were comparable at E15.5 (Fig. 2E and F). At E16.5, defects in the epididymis in $Pkd2^{-/-}$ mice became evident (Fig. 2C and D). The epididymal duct, derived from the Wolffian duct, started to coil in the caput segment of the

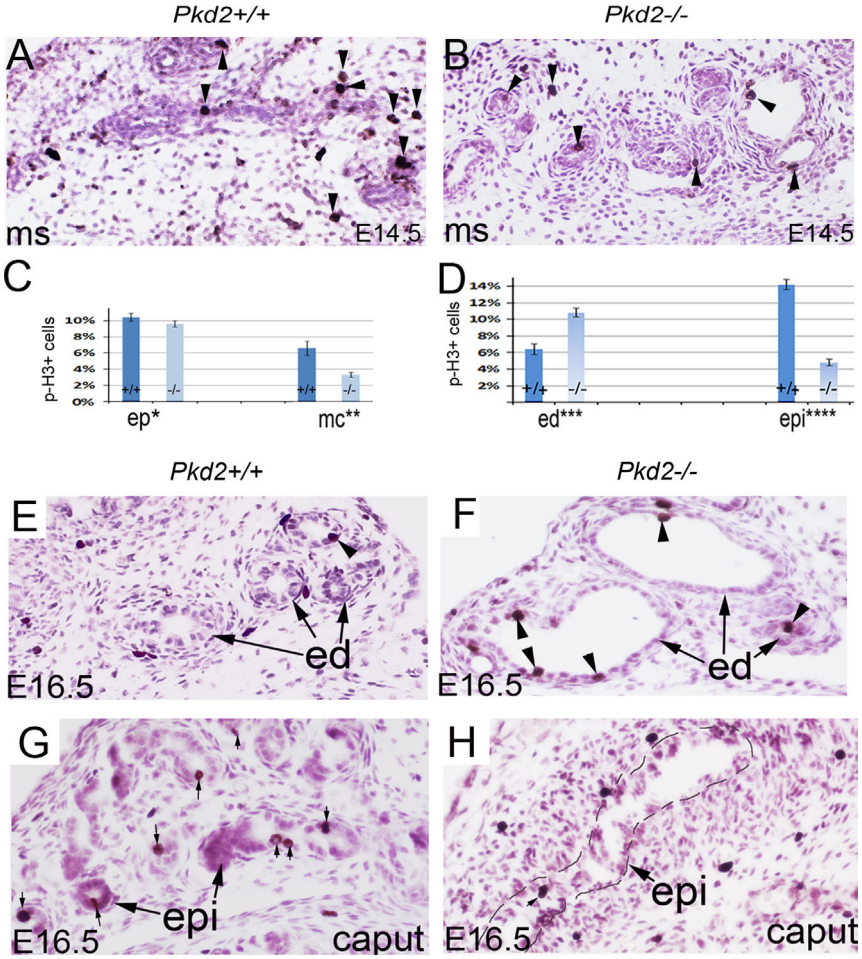

Fig. 3. Proliferation analysis in the male reproductive tract of $Pkd2^{-/-}$ mice. (A, B) Phospho-histone H3 staining of E14.5 mesonephros, arrow heads indicate positive cells. (C) Bar graphs represent mitotic ratios in the mesonephros. (D) Bar graphs represent mitotic ratios in the efferent ducts and caput epididymis. Data are expressed as mean and SEM. (E, F) Phospho-histone H3 staining of E16.5 efferent ducts, arrow heads indicate positive cells. (G, H) Phospho-histone H3 staining of the epididymis, arrows indicate positive cells. *non-significant, $^{**}P < 0.01$, $^{***}P < 0.01$, $^{****}P < 0.01$. cap: caput epididymis; ed: efferent duct; ep: epithelium; epi: epididymis; mc: mesenchyme; ms: mesonephros.

wild-type epididymis at this time. By contrast, it remained straight in the mutant epididymis, indicating a delay in development (Fig. 2C and D).

We performed apoptosis and proliferation assays in the male reproductive tract. Staining with an antibody for phospho-histone H3, a mitosis marker, showed that mitosis in the E14.5 mesonephros was vigorous in both epithelium and mesenchyme in wild-type embryos but was reduced in the mutant mesonephros, notably in the mesenchyme, suggesting that *Pkd2* also regulates development of mesonephric mesenchyme (Fig. 3A–C). At E16.5, mitosis in the efferent ducts was less in control mice compared to the earlier time point, but remained active in mutants (Fig. 3D–F). In the epididymis, the mitotic ratio was significantly decreased in mutants compared to controls (Fig. 3D, G, H). TUNEL staining failed to detect apoptosis in the efferent ducts and epididymis in both genotypes (data not shown). Altogether, these data suggest that *Pkd2* plays an essential role in male reproductive tract development.

3.3. Epithelial-specific disruption of Pkd2 is sufficient to cause efferent duct dilation and epididymis coiling defect in the male reproductive tract

To dissect roles of *Pkd2* in different cell types, we used *Pax2-cre* mice to specifically disrupt *Pkd2* from epithelium of the male reproductive tract and examined male reproductive tract development in *Pax2-cre; Pkd2*$^{Null/Loxp}$ conditional knockout mice. As it has been shown in previous studies, *Pax2-cre* specifically targets epithelium of the reproductive tract, but has no effect on mesenchyme and the testis (Nie and Arend, 2013). Therefore, using this model, we could exclude a potential effect from testis defects present in *Pkd2*$^{-/-}$ mice. Indeed, testis development in *Pax2-cre; Pkd2*$^{Null/Loxp}$ mice was comparable to controls (Fig. 4A, B, E, F). Further, epithelial disruption of *Pkd2* did not appear to affect androgen levels since descent of the testes was normal and appearance of the external genitalia was indistinguishable in mutant versus wild-type mice (Fig. 4A and B, data not shown).

Pax2-cre; Pkd2$^{Null/Loxp}$ mutant mice died in the first few days after birth with severe kidney dilation. In the reproductive tract, efferent duct dilation and lack of epididymal coiling in conditional knockout mice were similar to that seen in *Pkd2*$^{-/-}$ mice. Efferent duct dilation was first visible at E15.5 (data not shown), and became very severe at late gestation and postnatal stages (Fig. 4A–F). Epithelial coiling events in the mutant epididymis were very few when examined at the newborn stage, whereas a highly coiled epithelial tube was seen in the control epididymis at this time (Fig. 4C and D).

Phospho-histone H3 staining showed that, in the efferent ducts, the mitotic ratios in mutants and controls were comparable at the newborn stage (Fig. 4G, H, K). In the epididymis, however, mitotic activity was very low in mutants but remained abundant in controls (Fig. 4I–K).

In postnatal stages, we also found a marked delay in seminal vesicle morphogenesis in mutant mice (Fig. 4L and M). Normally, the seminal vesicle bud starts to elongate and branch during postnatal development. However, seminal vesicle development in mutants was arrested at the bud stage (Fig. 4L and M). Collectively, these results show that epithelial expression of *Pkd2* plays a pivotal role for male reproductive tract development.

3.4. Epithelial deletion of Pkd2 causes changes in cellular phenotype and disrupts epithelial integrity in the male reproductive tract

The conditional knockout mouse model allows us to analyze reproductive tract development throughout embryogenesis and also during early postnatal development. We examined mutant epithelium with a set of molecular markers, including pan-cytokeratin, α-tubulin and laminin. During postnatal development,

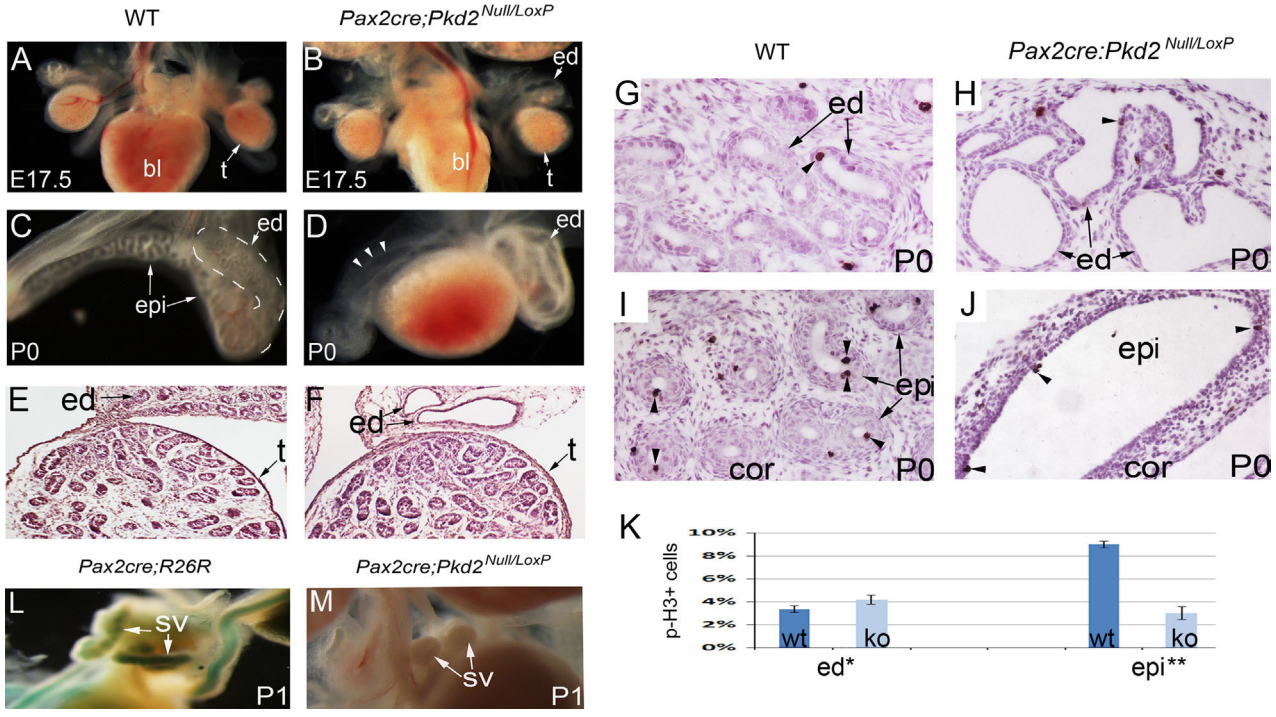

Fig. 4. Reproductive tract defects in *Pax2-cre; Pkd2*$^{Null/Loxp}$ mice. (A, B) The male reproductive system at E17.5, showing efferent duct dilation in the mutant. (C, D) The male reproductive system at Po, showing efferent duct dilation and epididymal coiling defect in the mutant. Mutant epididymis was also dilated as indicated by arrow heads. (E, F) Histology of the testes and efferent ducts at P0. (G, H) Phospho-histone3 staining of the efferent ducts. Arrow heads indicate mitotic cells. (I, J) Phospho-histone3 staining of the epididymis. Arrow heads indicate mitotic cells. (K) Bar graphs represent mitotic ratios in the efferent ducts and epididymides. (L, M) Delayed seminal vesicle development in the mutant at P1. *: non-significant; **: $P < 0.01$. bl: bladder; cor: corpus epididymis; ed: efferent duct; epi: epididymis; ko: knockout; p-H3: phospho-histone H3; sv: seminal vesicle; t: testis; wt: wild type.

epithelial cells in the efferent ducts became flat rather than cuboidal, as highlighted by pan-cytokeratin staining (Fig. 5A and B). Epithelial cells of cysts were often separated from one another, suggesting loss of cell–cell adhesions (Fig. 5A and B). Cell shape change in *Pkd2*$^{-/-}$ epithelia suggests that *Pkd2* might be involved in regulating cytoskeleton dynamics. We examined tubulin cytoskeleton in the reproductive tract of *Pax2-cre; Pkd2*$^{Null/Loxp}$ mutant mice by immunostaining for α-tubulin, a subunit for microtubule assembly. We found that α-tubulin levels were reduced in the mutant efferent ducts and epididymis during postnatal development (Fig. 5C–F). Changes in the basement membranes, visualized by laminin staining, were also detected in mutant epithelia (Fig. 5G–J). Laminin staining in mutant mice showed thickened, layered and often discontinuous basement membranes (Fig. 5H and J). Altogether, these results suggest that epithelial PC-2 plays an essential role in maintaining epithelial integrity.

3.5. Epithelial-specific deletion of Pkd2 affects Tgf-β/Bmp and canonical Wnt signaling in the male reproductive tract

To gain a mechanistic understanding of the reproductive tract defects in PC-2 deficiency mice, we analyzed a set of developmental signaling pathways that are critical for male reproductive tract development. The *Tgf-β/Bmp* signaling pathways are critical for ductal system development in the male reproductive tract, and disrupting *Tgf-β* signaling abolishes epithelial coiling in the epididymis (Di Giovanni et al., 2011; Hu et al., 2004; Tomaszewski et al., 2007). We therefore tested if epithelial disruption of *Pkd2* affects *Tgf-β/Bmp* signaling in the male reproductive tract by immunostaining for phospho-Smad2 and phospho-Smad1/5, which are intracellular mediators of *Tgf-β* and *Bmp* signaling respectively. Consistent with a previous study (Tomaszewski et al., 2007), the level of phospho-Smad2 was high in wild-type epididymal

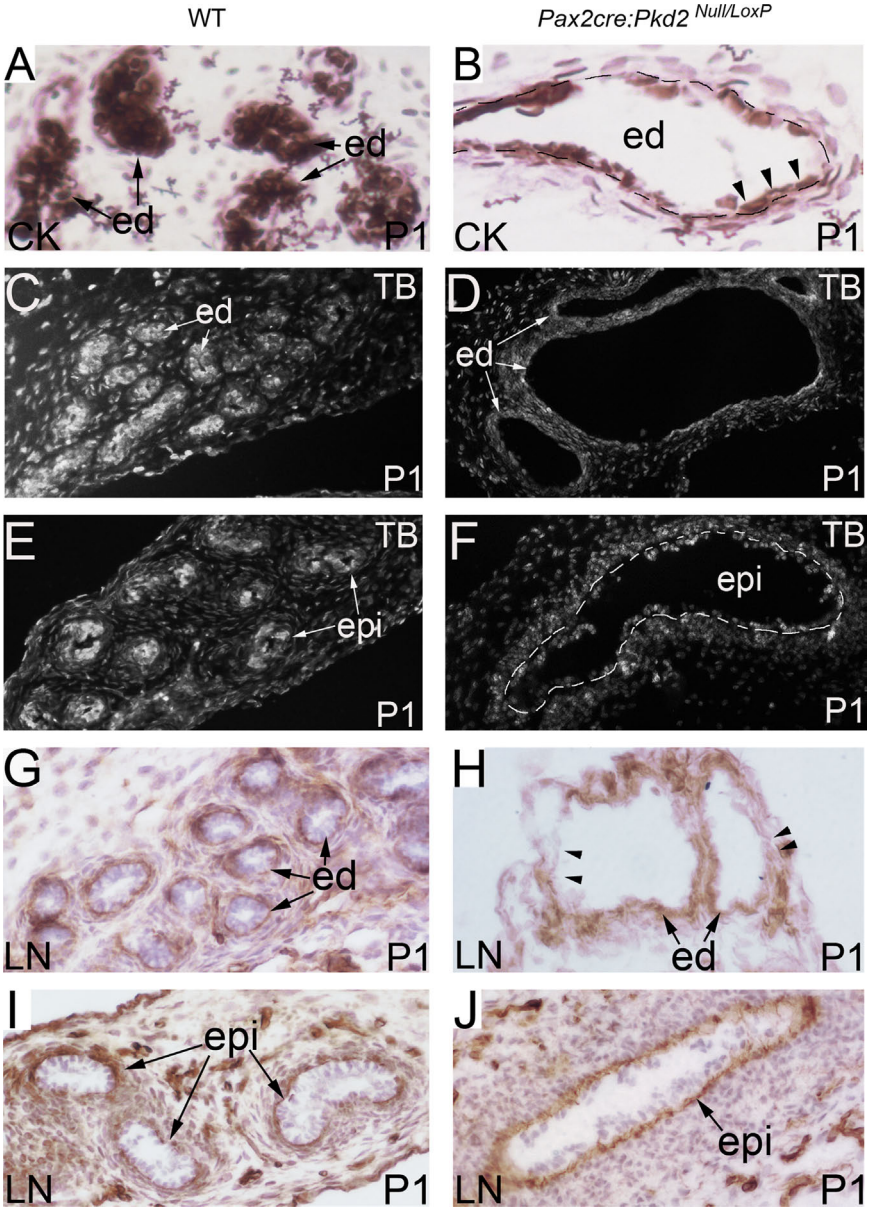

Fig. 5. Pan-cytokeratin, laminin and α-tubulin staining in the male reproductive tract of *Pax2-cre; Pkd2*$^{Null/Loxp}$ mice. (A, B) Pan-cytokeratin staining of the efferent ducts at P1. Cystic epithelium exhibits a flattened appearance rather than cuboidal in the mutant as indicated by arrow heads. (C, D) α- tubulin staining of the efferent ducts at P1. (E, F) α- tubulin staining of the epididymis at P1. (G, H) Laminin staining of the efferent ducts at P1. Arrow heads indicate areas lacking laminin staining. (I, J) Laminin staining of the epididymis at P1. CK: pan-cytokeratin; cor: corpus epididymis; ed: efferent duct; epi: epididymal epithelium; LN: laminin; TB: tubulin.

epithelium, indicative of high requirements of *Tgf-β* signaling by epithelial cells (Fig. 6A). However, in mutant epididymal epithelium, the phospho-Smad2 level was visibly reduced beginning at late gestation, notably in the caput and corpus segments (Fig. 6B and data not shown). Similarly, the level of phospho-Smad2 was reduced in the epithelium of the mutant efferent ducts (Fig. 6C and D). Staining for phospho-Smad1/5 was reduced, suggesting that *Bmp* signaling was also compromised (Fig. 6E–I).These observations were further confirmed with western blot analysis (Fig. 6J).

Wnt signaling is critical for development of the Wolffian duct and its derivatives, and upregulation of canonical *Wnt* signaling was found in polycystic kidney disease (Carroll et al., 2005; Kim et al., 2009; Lombardi et al., 2013). Here, we performed immunostaining for β-catenin, an essential intracellular mediator of canonical *Wnt* signaling, in the reproductive tract of *Pax2-cre; Pkd2*$^{Null/Loxp}$ mice. We found that β-catenin levels were dramatically reduced in the mutant epididymis, but significantly increased in the mutant efferent ducts (Fig. 6K–N). In cystic ductal epithelial cells, increased β-catenin expression was consistently detected (Fig. 6N). We further confirmed this observation by RT-PCR and real-time qRT-PCR assays (Fig. 6O and P). Changes in β-catenin levels suggest *Wnt* signaling might affect the mutant reproductive system. Altogether, these results suggest a link between PC-2 activity and critical developmental signaling pathways in the male reproductive tract.

3.6. Testis defects in *Pkd2*$^{-/-}$ mice

As PC-2 also showed robust expression during gonadogenesis, we thus examined testis development in *Pkd2*$^{-/-}$ mice. Histologic examination at E15.5 revealed atypical testicular cord morphology in mutant mice (Fig. 7A and B). We also detected a significant decrease in mitosis in the mutant testes at E16.5 with phospho-histone H3 staining (Fig. 7C–E). In agreement with decreased cell proliferation, testicular cord growth was retarded in mutants as indicated by a decrease in the average number of testicular cords per section (Fig. 7F). However, apoptosis assay by TUNEL staining did not reveal abnormal cell death in mutant testes at E13.5 and E16.5 (Fig. 7G–I; and data not shown). We further examined *Tgf-beta* signaling by immunostaining for phospho-Smad2, which is critical for testicular cord growth (Itman et al., 2006; Miles et al., 2013). Phospho-Smad2 was detected at a high level in the testicular cords of the controls (Fig. 7J). By contrast, the level of phospho-Smad2 was reduced in the mutant testicular cords (Fig. 7K). This was confirmed with western blot (Fig. 7L). Altogether, these results show that testicular defects exist in *Pkd2*$^{-/-}$ mice, and suggest that *Pkd2* plays a role in testis development. However, the severity of testis defects varied in *Pkd2*$^{-/-}$ mice.

Here, we also describe the testicular phenotype of *Pkd1*$^{-/-}$ mice, which has not been described previously. We observed a high level of *Pkd1* expression in the developing testes of *Pkd1*$^{LacZ/+}$ mice at E15.5, as indicated by *LacZ* staining (Fig. 8A). *Pkd1* expression was primarily found in the interstitial cells including the peritubular myoid cells, but was undetectable or barely detectable within the testicular cords (Fig. 8B). Testis morphology in *Pkd1*$^{-/-}$ mice was largely similar to that of *Pkd2*$^{-/-}$ mice. At E16.5, we found reduced interstitial tissue and a decrease in testicular cord growth (Fig. 8C–E). This is further supported by decreased mitosis in *Pkd1*$^{-/-}$ testes (Fig. 8F–H).

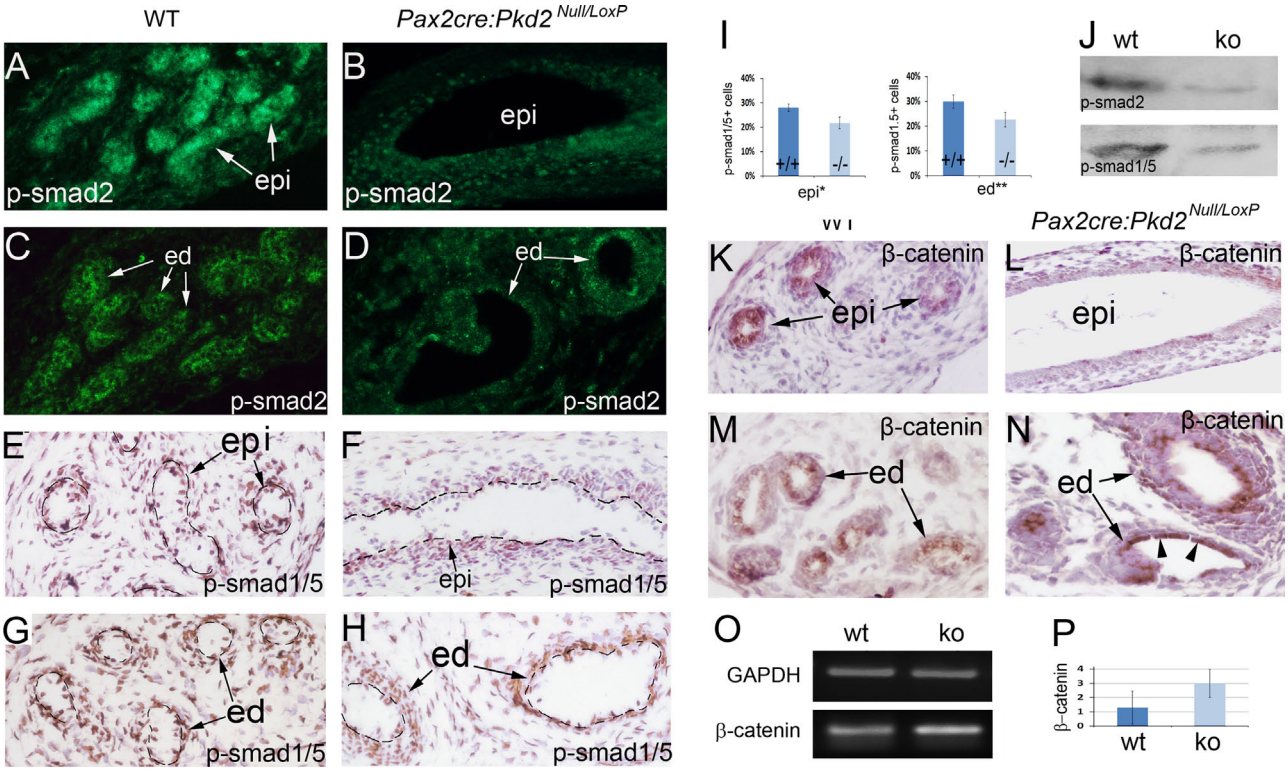

Fig. 6. Altered intracellular signal transduction of *Tgf-β/Bmp* and canonical *Wnt* signaling in the male reproductive tract of *Pax2-cre; Pkd2*$^{Null/Loxp}$ mice. (A, B) Immunofluorescence for phospho-Smad2 in the epididymis at P0. (C, D) Immunofluorescence for phospho-Smad2 in the efferent ducts at P0. (E, F) Immunohistochemistry for phospho-Smad1/5 in the epididymis at P0. (G, H) Immunohistochemistry for phospho-Smad1/5 in the efferent ducts at P0. (I) Bar graphs represent percentages of phospho-Smad1/5 positive cells in the epithelia. $^{*}P < 0.05$, and $^{**}P < 0.05$. (J) Western Blot for phospho-Smad2 and phospho-Smad1/5 of the epididymis and efferent ducts at P0. (K, L) β-Catenin staining of the epididymis at E18.5. (M, N) β-Catenin staining of the efferent ducts at E18.5. Note, significant upregulation of β-Catenin in the flat cells, as indicated by arrow heads. (O) RT-PCR for β-Catenin in E18.5 efferent ducts. (P) Relative fold expression of β-Catenin in E18.5 efferent ducts by real-time qRT-PCR. cor: corpus epididymis; ed: efferent duct; ko: knockout; epi: epididymis; wt: wild type.

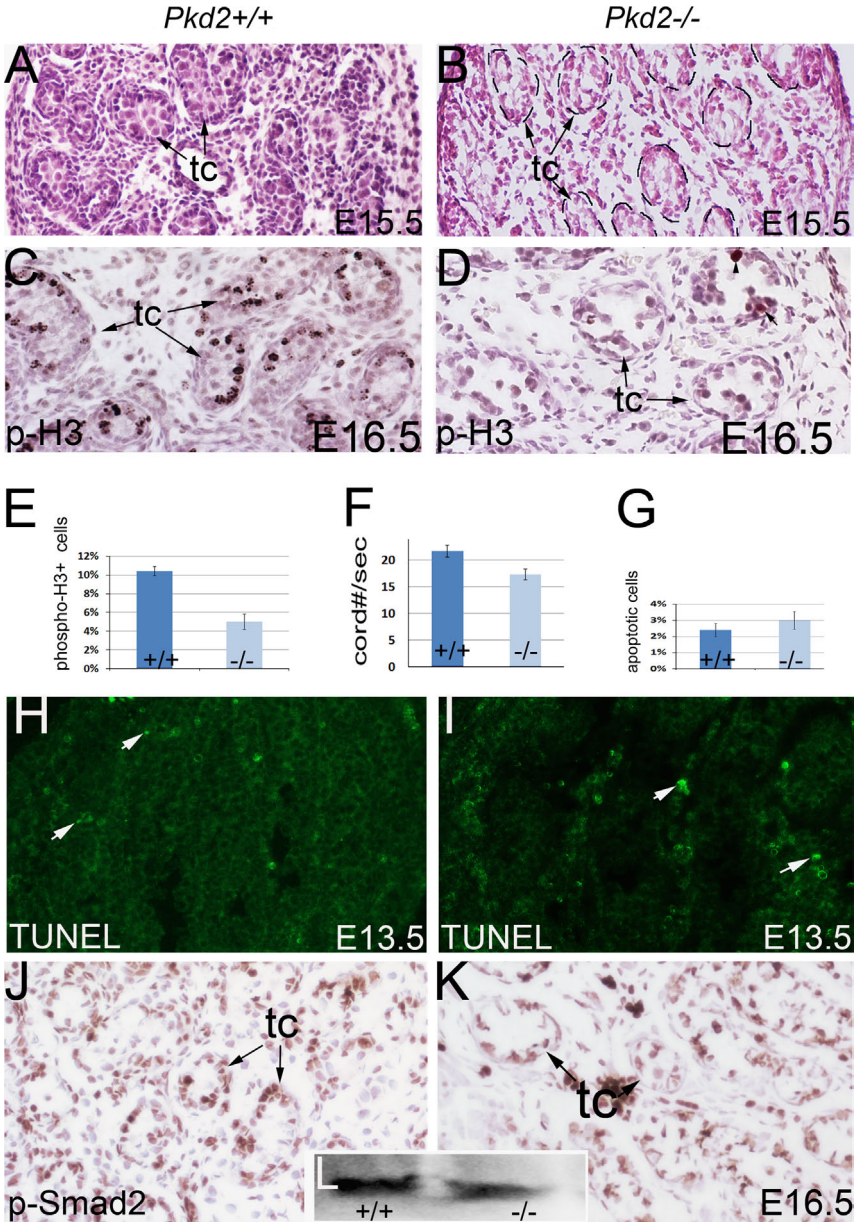

Fig. 7. Testis development in *Pkd2*$^{-/-}$ mice. (A, B) Histology of E15.5 testes. (C, D) Phospho-histone H3 staining of E16.5 testes. (E) Bar graphs represent mitotic ratios of the control and mutant testes (Mean ± SEM), $P < 0.05$. (F) Bar graphs represent average numbers of testicular cords per section (Mean ± SEM), $P < 0.05$. (G) Bar graphs represent ratios of apoptotic cells in the testes (Mean ± SEM). (H, I) TUNEL staining of E13.5 testes. (J, K) Phospho-Smad2 staining of the testes. (L) Western Blot for phospho-Smad2 of the testes. p-H3: phospho-histone H3; tc: testicular cord.

4. Discussion

This work demonstrates for the first time that *Pkd2*, like *Pkd1*, is essential for male reproductive system development. Disruption of *Pkd2* in mice causes efferent duct dilation, lack of epididymal coiling, and altered testicular development. Specific deletion of *Pkd2* from the epithelium is sufficient to cause efferent duct and epididymis defects recapitulating those seen in *Pkd2*$^{-/-}$ mice, suggesting that epithelial expression of *Pkd2* plays a critical role in maintaining epithelial integrity in the male reproductive tract. Our results also show that *Pkd2* function is not critical for early tubulogenesis in the mesonephros but is essential for maintaining tubule integrity. Loss of *Pkd2* induces phenotypic changes in epithelial cells and basement membrane malformation in the efferent ducts. Elevated β-catenin levels are a key feature of *Pkd2*-null epithelial cells of the efferent ducts, which explains increased epithelial cell proliferation in mutant efferent ducts.

Multiple lines of evidence suggest that ADPKD genes regulate cell proliferation (Aguiari et al., 2008; Nishio et al., 2005). It has been proposed that increased cell proliferation induced by ADPKD gene mutations is an important mechanism for cyst formation in the kidney (Chapin and Caplan, 2010; Hanaoka and Guggino, 2000; Wilson, 2004). In the male reproductive system, however, we detected contrasting effects of *Pkd2* on mitosis in different tissues. While loss of *Pkd2* did not limit cell proliferation in the developing efferent ducts, it slowed proliferation in the epididymis and testis. In the epididymis, epithelial cell proliferation is controlled by testosterone signaling from the testis, which is further relayed by local mesenchymal signaling (Cooke et al., 1991; Cornwall, 2009; Tomaszewski et al., 2007). Our data suggest that PC-2 activity is critical for maintaining the testosterone signaling cascade by linking to developmental signaling pathways that control epithelial development. Indeed, deficiency of PC-2 impaired both *Tgf-β/Bmp* and *Wnt/β-Catenin* signaling pathways in the epididymis, which are essential for epithelial development

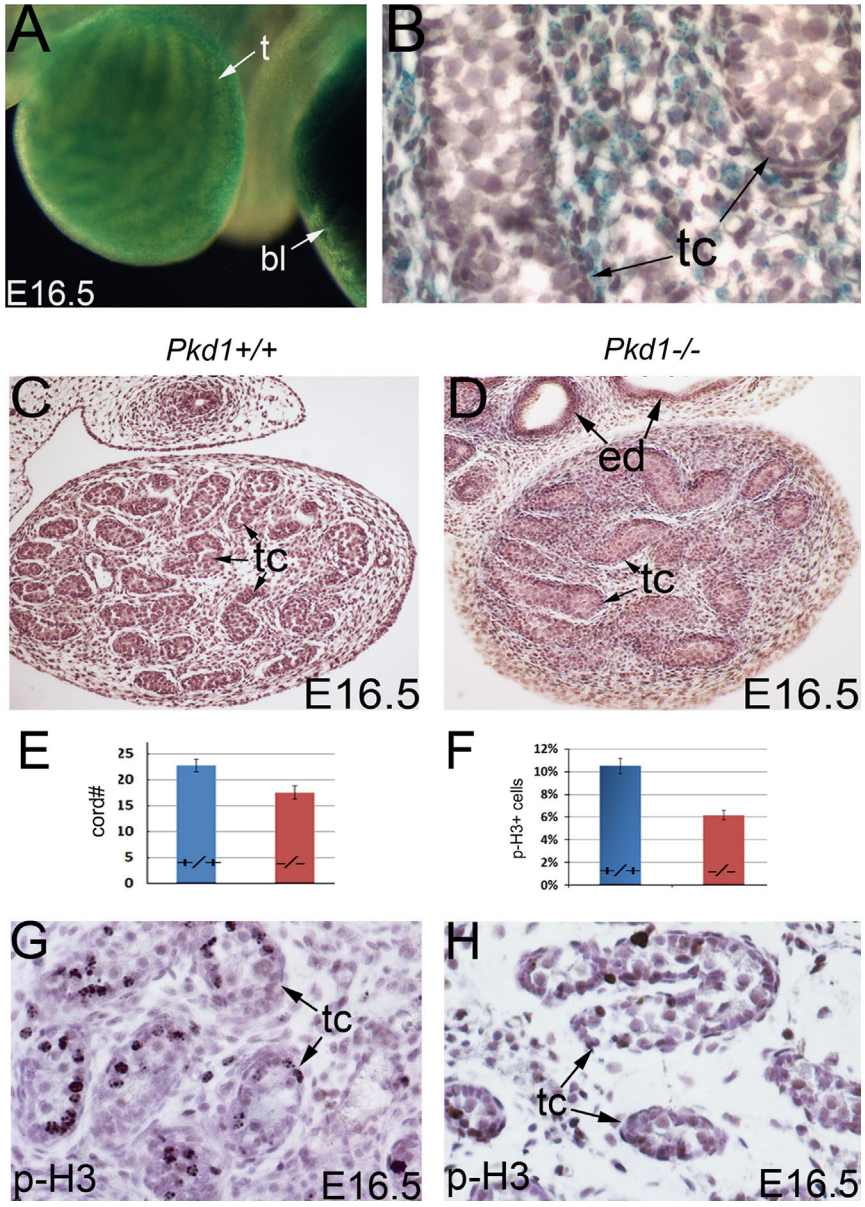

Fig. 8. *Pkd1* expression and testis development in $Pkd1^{-/-}$ mice. (A) LacZ staining of $Pkd1^{LacZ/+}$ testis at E16.5. (B) Section of LacZ-stained testis, showing widespread *Pkd1* expression in the interstitium. (C, D) Histology of E16.5 testes. (E) Bar graphs represent average testicular cord numbers per section of each genotype (Mean ± SEM), $P < 0.05$. (F) Bar graphs represent proliferation ratios (mean ± SEM), $P < 0.05$. (G, H) Phospho-histone H3 staining of the testes. bl: bladder; ed: efferent duct; p-H3: phospho-histone H3; t: testis; tc: testicular cord.

(Di Giovanni et al., 2011; Hu et al., 2004; Lombardi et al., 2013; Tomaszewski et al., 2007; Zhao et al., 2001). Altogether, these data suggest that the functions of *Pkd2* for cell signaling and proliferation depend on a specific developmental context.

How PC-2 activity regulates these developmental pathways remains to be determined at the molecular level. Besides an established role in regulating calcium influx, PC-2 also regulates cytoskeletal dynamics. It has been shown that PC-2 is involved in actin-cytoskeleton regulation via interaction with Hax-1 (Gallagher et al., 2000). Here, we show that PC-2 activity is also important for maintenance of tubulin, an essential component for microtubule assembly. One possible mechanism is that PC-2 regulates developmental signaling through maintaining the microtubules. Indeed, a recent study showed that microtubule function is critical for *Tgf-β* signaling in a developmental process (Kitase and Shuler, 2013). Microtubules are also the components of cytoskeleton for the cilium, an organelle important for cell function in a physiological setting. Microtubule reduction also indicates that cilia might change in *Pkd2* mutant epithelia. Overall, the reproductive tract phenotype of $Pkd2^{-/-}$ mice is largely similar to that of $Pkd1^{-/-}$ mice, suggesting that PC-2 and PC-1 may regulate common cellular activities during ductal system development of the male reproductive system (Nie and Arend, 2013).

In this work, we also revealed novel roles of *Pkd2* and *Pkd1* in testicular development. We found high levels of *Pkd1* and *Pkd2* expression in testicular interstitial tissue, and detected altered testis development in *Pkd1* and *Pkd2* null mice. Besides decreased interstitial cell numbers, the function of the interstitial cells might also be impaired in mutant mice, given the altered levels of phospho-Smad2. These changes may explain delayed development of the testicular cords in the mutant mice. In addition, we also detected robust expression of PC-2 in PGCs during cord morphogenesis, which is not found for *Pkd1*/PC-1. *Pkd2* is important for germ cell maturation and motility (Gao et al., 2003). However, the functional role of *Pkd2* in early PGC development

remains to be elucidated. It is possible that PC-2 is involved in PGC clustering and proper colonization in the testicular cords, as we often saw abnormal histology of testicular cords in *Pkd2*$^{-/-}$ mice.

Finally, we also found that *Pkd2* is required for normal seminal vesicle development. Seminal vesicle cysts are the most common finding among the reproductive tract cysts in ADPKD patients (Belet et al., 2002; Danaci et al., 1998). Therefore, PKD genes are likely critical for both development and maintenance of the seminal vesicles. It should be noted that the male reproductive system continues to develop and mature until puberty. Maintenance of reproductive organs may also require PKD genes in adulthood. An inducible knockout system may prove useful to address complex roles of PKD genes in maturation and/or maintenance of the adult male reproductive system.

Conflict of interest statement

Authors declare that there are no conflicts of interest.

Acknowledgments:

This work was supported by a generous donation from the Lillian Goldman Charitable Trust. This study utilized resources provided by the NIDDK sponsored Johns Hopkins Polycystic Kidney Disease Research and Clinical Core Center (P30 DK090868). We are grateful to Dr. Timothy H. Moran of the Department of Psychiatry and Behavioral Sciences at Johns Hopkins University School of Medicine for use of the microscope and imaging system. We thank Dr. Andy Groves at the Baylor College of Medicine and Dr. Angelika Doetzlhofer of the Department of Neuroscience at Johns Hopkins University School of Medicine for providing *Pax2-cre* mice.

References

Aguiari, G., Trimi, V., Bogo, M., Mangolini, A., Szabadkai, G., Pinton, P., Witzgall, R., Harris, P.C., Borea, P.A., Rizzuto, R., del Senno, L., 2008. Novel role for polycystin-1 in modulating cell proliferation through calcium oscillations in kidney cells. Cell Prolif. 41, 554–573.

Bataille, S., Demoulin, N., Devuyst, O., Audrezet, M.P., Dahan, K., Godin, M., Fontes, M., Pirson, Y., Burtey, S., 2011. Association of PKD2 (polycystin 2) mutations with left-right laterality defects. American J. Kidney Dis. 58, 456–460.

Belet, U., Danaci, M., Sarikaya, S., Odabas, F., Utas, C., Tokgoz, B., Sezer, T., Turgut, T., Erdogan, N., Akpolat, T., 2002. Prevalence of epididymal, seminal vesicle, prostate, and testicular cysts in autosomal dominant polycystic kidney disease. Urology 60, 138–141.

Bhunia, A.K., Piontek, K., Boletta, A., Liu, L., Qian, F., Xu, P.N., Germino, F.J., Germino, G.G., 2002. PKD1 induces p21(waf1) and regulation of the cell cycle via direct activation of the JAK-STAT signaling pathway in a process requiring PKD2. Cell 109, 157–168.

Brennan, J., Capel, B., 2004. One tissue, two fates: molecular genetic events that underlie testis versus ovary development. Nat. Rev. Genet. 5, 509–521.

Cai, Y., Maeda, Y., Cedzich, A., Torres, V.E., Wu, G., Hayashi, T., Mochizuki, T., Park, J.H., Witzgall, R., Somlo, S., 1999. Identification and characterization of polycystin-2, the PKD2 gene product. J. Biol. Chem. 274, 28557–28565.

Carroll, T.J., Park, J.S., Hayashi, S., Majumdar, A., McMahon, A.P., 2005. Wnt9b plays a central role in the regulation of mesenchymal to epithelial transitions underlying organogenesis of the mammalian urogenital system. Dev. Cell 9, 283–292.

Casuscelli, J., Schmidt, S., DeGray, B., Petri, E.T., Celic, A., Folta-Stogniew, E., Ehrlich, B.E., Boggon, T.J., 2009. Analysis of the cytoplasmic interaction between polycystin-1 and polycystin-2. Am. J. Physiol. Ren. Physiol. 297, F1310–F1315.

Chapin, H.C., Caplan, M.J., 2010. The cell biology of polycystic kidney disease. J. Cell Biol. 191, 701–710.

Chauvet, V., Qian, F., Boute, N., Cai, Y., Phakdeekitacharoen, B., Onuchic, L.F., Attie-Bitach, T., Guicharnaud, L., Devuyst, O., Germino, G.G., Gubler, M.C., 2002. Expression of PKD1 and PKD2 transcripts and proteins in human embryo and during normal kidney development. Am. J. Pathol. 160, 973–983.

Combes, A.N., Wilhelm, D., Davidson, T., Dejana, E., Harley, V., Sinclair, A., Koopman, P., 2009. Endothelial cell migration directs testis cord formation. Dev. Biol. 326, 112–120.

Cooke, P.S., Young, P., Cunha, G.R., 1991. Androgen receptor expression in developing male reproductive organs. Endocrinology 128, 2867–2873.

Cornwall, G.A., 2009. New insights into epididymal biology and function. Hum. Reprod. Update 15, 213–227.

Danaci, M., Akpolat, T., Bastemir, M., Sarikaya, S., Akan, H., Selcuk, M.B., Cengiz, K., 1998. The prevalence of seminal vesicle cysts in autosomal dominant polycystic kidney disease. Nephrol., Dial., Transplant. 13, 2825–2828.

Di Giovanni, V., Alday, A., Chi, L., Mishina, Y., Rosenblum, N.D., 2011. Alk3 controls nephron number and androgen production via lineage-specific effects in intermediate mesoderm. Development 138, 2717–2727.

Gallagher, A.R., Cedzich, A., Gretz, N., Somlo, S., Witzgall, R., 2000. The polycystic kidney disease protein PKD2 interacts with Hax-1, a protein associated with the actin cytoskeleton. Proc. Natl. Acad. Sci. U.S.A 97, 4017–4022.

Gao, Z., Ruden, D.M., Lu, X., 2003. PKD2 cation channel is required for directional sperm movement and male fertility. Curr. Biol. 13, 2175–2178.

Garcia-Gonzalez, M.A., Outeda, P., Zhou, Q., Zhou, F., Menezes, L.F., Qian, F., Huso, D.L., Germino, G.G., Piontek, K.B., Watnick, T., 2010. Pkd1 and Pkd2 are required for normal placental development. PloS One 16 (5(9)), e12821, http://dx.doi.org/10.1371/journal.pone.0012821.

Gilbert-Sirieix, M., Makoukji, J., Kimura, S., Talbot, M., Caillou, B., Massaad, C., Massaad-Massade, L., 2011. Wnt/beta-catenin signaling pathway is a direct enhancer of thyroid transcription factor-1 in human papillary thyroid carcinoma cells. PloS One 6, e22280.

Gonzalez-Perrett, S., Kim, K., Ibarra, C., Damiano, A.E., Zotta, E., Batelli, M., Harris, P.C., Reisin, I.L., Arnaout, M.A., Cantiello, H.F., 2001. Polycystin-2, the protein mutated in autosomal dominant polycystic kidney disease (ADPKD), is a Ca2^{+}-permeable nonselective cation channel. Proc. Natl. Acad. Sci. U.S.A 98, 1182–1187.

Hanaoka, K., Guggino, W.B., 2000. cAMP regulates cell proliferation and cyst formation in autosomal polycystic kidney disease cells. J. Am. Soc. Nephrol. 11, 1179–1187.

Hannema, S.E., Hughes, I.A., 2007. Regulation of Wolffian duct development. Horm. Res. 67, 142–151.

Herpin, A., Schartl, M., 2011. Sex determination: switch and suppress. Curr. Biol. 21, R656–R659.

Hu, J., Chen, Y.X., Wang, D., Qi, X., Li, T.G., Hao, J., Mishina, Y., Garbers, D.L., Zhao, G.Q., 2004. Developmental expression and function of Bmp4 in spermatogenesis and in maintaining epididymal integrity. Dev. Biol. 276, 158–171.

Itman, C., Mendis, S., Barakat, B., Loveland, K.L., 2006. All in the family: TGF-beta family action in testis development. Reproduction 132, 233–246.

Joseph, A., Yao, H., Hinton, B.T., 2009. Development and morphogenesis of the Wolffian/epididymal duct, more twists and turns. Dev. Biol. 325, 6–14.

Kanagarajah, P., Ayyathurai, R., Lynne, C.M., 2011. Male infertility and adult polycystic kidney disease - revisited: case report and current literature review. Andrologia 44 (Suppl 1), 838–841.

Karl, J., Capel, B., 1998. Sertoli cells of the mouse testis originate from the coelomic epithelium. Dev. Biol. 203, 323–333.

Kim, I., Ding, T., Fu, Y., Li, C., Cui, L., Li, A., Lian, P., Liang, D., Wang, D.W., Guo, C., Ma, J., Zhao, P., Coffey, R.J., Zhan, Q., Wu, G., 2009. Conditional mutation of Pkd2 causes cystogenesis and upregulates beta-catenin. J. Am. Soc. Nephrol. 20, 2556–2569.

Kitase, Y., Shuler, C.F., 2013. Microtubule disassembly prevents palatal fusion and alters regulation of the E-cadherin/catenin complex. Int. J. Dev. Biol. 57, 55–60.

Koulen, P., Cai, Y., Geng, L., Maeda, Y., Nishimura, S., Witzgall, R., Ehrlich, B.E., Somlo, S., 2002. Polycystin-2 is an intracellular calcium release channel. Nat. Cell Biol. 4, 191–197.

Lombardi, A.P., Royer, C., Pisolato, R., Cavalcanti, F.N., Lucas, T.F., Lazari, M.F., Porto, C.S., 2013. Physiopathological aspects of the Wnt/beta-catenin signaling pathway in the male reproductive system. Spermatogenesis 3, e23181.

Manno, M., Marchesan, E., Tomei, F., Cicutto, D., Maruzzi, D., Maieron, A., Turco, A., 2005. Polycystic kidney disease and infertility: case report and literature review. Arch. Ital. Urol., Androl. 77, 25–28.

Markowitz, G.S., Cai, Y., Li, L., Wu, G., Ward, L.C., Somlo, S., D'Agati, V.D., 1999. Polycystin-2 expression is developmentally regulated. Am. J. Physiol. 277, F17–F25.

Martineau, J., Nordqvist, K., Tilmann, C., Lovell-Badge, R., Capel, B., 1997. Male-specific cell migration into the developing gonad. Curr. Biol. 7, 958–968.

Miles, D.C., Wakeling, S.I., Stringer, J.M., van den Bergen, J.A., Wilhelm, D., Sinclair, A.H., Western, P.S., 2013. Signaling through the TGF beta-activin receptors ALK4/5/7 regulates testis formation and male germ cell development. PloS One 8, e54606.

Molyneaux, K., Wylie, C., 2004. Primordial germ cell migration. Int. J. Dev. Biol. 48, 537–544.

Nie, X., Arend, L.J., 2013. Pkd1 is required for male reproductive tract development. Mech. Dev. 130, 567–576.

Nishio, S., Hatano, M., Nagata, M., Horie, S., Koike, T., Tokuhisa, T., Mochizuki, T., 2005. Pkd1 regulates immortalized proliferation of renal tubular epithelial cells through p53 induction and JNK activation. J. Clin. Investig. 115, 910–918.

Ohyama, T., Groves, A.K., 2004. Generation of Pax2-Cre mice by modification of a Pax2 bacterial artificial chromosome. Genesis 38, 195–199.

Qian, F., Germino, F.J., Cai, Y., Zhang, X., Somlo, S., Germino, G.G., 1997. PKD1 interacts with PKD2 through a probable coiled-coil domain. Nat. Genet. 16, 179–183.

Tomaszewski, J., Joseph, A., Archambeault, D., Yao, H.H., 2007. Essential roles of inhibin beta A in mouse epididymal coiling. Proc. Natl. Acad. Sci. U.S.A 104, 11322–11327.

Torra, R., Sarquella, J., Calabia, J., Marti, J., Ars, E., Fernandez-Llama, P., Ballarin, J., 2008. Prevalence of cysts in seminal tract and abnormal semen parameters in patients with autosomal dominant polycystic kidney disease. Clin. J. Am. Soc. Nephrol. 3, 790–793.

Tsiokas, L., Kim, S., Ong, E.C., 2007. Cell biology of polycystin-2. Cell. Signal. 19, 444–453.
Vora, N., Perrone, R., Bianchi, D.W., 2008. Reproductive issues for adults with autosomal dominant polycystic kidney disease. Am. J. Kidney Dis. 51, 307–318.
Wilson, P.D., 2001. Polycystin: new aspects of structure, function, and regulation. J. Am. Soc. Nephrol. 12, 834–845.
Wilson, P.D., 2004. Polycystic kidney disease. New Engl. J. Med. 350, 151–164.
Wu, G., Markowitz, G.S., Li, L., D'Agati, V.D., Factor, S.M., Geng, L., Tibara, S., Tuchman, J., Cai, Y., Park, J.H., van Adelsberg, J., Hou Jr., H., Kucherlapati, R., Edelmann, W., Somlo, S., 2000. Cardiac defects and renal failure in mice with targeted mutations in Pkd2. Nat. Genet. 24, 75–78.
Xu, G.M., Gonzalez-Perrett, S., Essafi, M., Timpanaro, G.A., Montalbetti, N., Arnaout, M.A., Cantiello, H.F., 2003. Polycystin-1 activates and stabilizes the polycystin-2 channel. J. Biol. Chem. 278, 1457–1462.
Yoder, B.K., Hou, X., Guay-Woodford, L.M., 2002. The polycystic kidney disease proteins, polycystin-1, polycystin-2, polaris, and cystin, are co-localized in renal cilia. J. Am. Soc. Nephrol. 13, 2508–2516.
Yoshiba, S., Shiratori, H., Kuo, I.Y., Kawasumi, A., Shinohara, K., Nonaka, S., Asai, Y., Sasaki, G., Belo, J.A., Sasaki, H., Nakai, J., Dworniczak, B., Ehrlich, B.E., Pennekamp, P., Hamada, H., 2012. Cilia at the node of mouse embryos sense fluid flow for left-right determination via Pkd2. Science 338, 226–231.
Zhao, G.Q., Chen, Y.X., Liu, X.M., Xu, Z., Qi, X., 2001. Mutation in Bmp7 exacerbates the phenotype of Bmp8a mutants in spermatogenesis and epididymis. Dev. Biol. 240, 212–222.